REMARKS

This Response is being made following a telephone interview with the Examiner, which occurred on 13 June 2006. The amendments above, and the following remarks are in accordance with the understandings regarding allowable subject matter, as detailed in said communication. Reconsideration of the above-identified application in view of the amendments above and the remarks following is respectfully requested.

Claims 1-260 are in this case. Claims 1-94, and 123-260 were withdrawn under a restriction requirement as drawn to a non-elected invention. Claims 95-122 have been rejected. Claims 102 and 120 have now been cancelled without prejudice, rendering moot the Examiner's rejection thereof. Claims 95, 114, 115, 117-118, 121 and 122 have now been amended.

Priority

The Examiner has required to show how the instant application differs from the parent application 09/915,527, in order to properly select priority.

The present application is a Continuation-in-Part of PCT/IL02/00398, filed May 21, 2002, which claims priority from U.S. Patent Application No. 09/915,527, filed July 27, 2001, now abandoned and U.S. Provisional Patent Application No. 60/292,953, filed May 24, 2001, now expired. The present application differs from 09/915,527 in disclosure of (1) use of recycled esterase (lipase) (Example 5, Fig. 7); (2) use of recycled emulsifier (deoxycholate) (Example 6, Figs 9 and 10); (3) alkaline extraction with ethyl acetate (Example 6, Figs. 9 and 10); (4) methods for Vitamin E removal and enrichment from deesterified carotenoids (Example 7, Figs. 13 and 14); (5) demonstration of superior antioxidant effects of the deesterified carotenoids (Example 8, Figs. 15 and 16); (6) demonstration of superior stability of the deesterified carotenoids of the present invention (Example 9, Fig. 12).

35 U.S.C. § 102(a), Rejections

The Examiner has rejected claims 95, 96, 98, 100, 101, 103, 109-111 and 120 under 35 USC 102(a) as being anticipated by Breithaupt (Z fur Naturforsch. 2000 55:971-75). The Examiner's rejections are respectfully traversed. Claims 102 and 120 have now been cancelled, rendering moot the Examiner's rejection thereof. Claims 95, 114, 115, 117-118, 121 and 122 has now been amended.

The Examiner states that Breithaupt teaches red pepper extracts treated with lipase to free carotenoids, that the samples are extracted with ethyl acetate, combined with buffer, bile salts and $CaCl_2$, then lipase with $CaCl_2$ added and incubated, and concludes that all of the claimed features of claims 95, 96, 98, 100, 101, 103, 109-111 and 120 are taught by Breithaupt.

Applicant wishes to point out that the invention as claimed in claims 95, 96, 98, 100, 101, 103, 109-111 and 120 is of methods of increasing a fraction of free carotenoids in a source of fatty acid-esterified carotenoids, using enzymatic deesterification of the fatty acid carotenoids, using a recycled emulsifier. Whereas the methods of the present invention result in superior enrichment of the free carotenoid fraction, and a near-total reduction of the mono- and diester forms of carotenoids, as pointed out in the telephone interview of June 13, 2006, the methods taught by Brehaupt et al fail to reduce the contamination with mono- and diester carotenoiods.

The failure of the method described by Breithaupt to reduce the contamination of the carotenoid product with mono- and diesterified carotenoids is by no means trivial. Extensive efforts to improve the efficiency of hydrolysis, and reduce esterified derivatives of carotenoids, proved unfruitful:

> "Our investigations showed, that the concentrations of calcium and bile salts, temperature, and equilibration time do not change the degree of hydrolysis."(page 975, left column).

Thus, Breithaupt was aware of the need for improved conditions of hydrolysis, and attempted varying nearly all the reaction conditions in order to increase the free carotenoid fraction of the prior art preparation. Manipulation of the enzyme:substrate ratio and enzyme source yielded similar disappointing results:

> "...Various concentrations of lipase were applied. However, higher concentrations of lipase did not enhance carotenoid hydrolysis. Lipase from porcine pancreas...gave generally lower yields."(page 975, left column).

Breithaupt provides an interpretation of the persistence of mono- and diesterified derivatives of the red-pepper carotenoid capsanthin, making reference to other sudies having encountered similar results:

> "This supports the assumption, that neither diesterified nor monoesterified carotenoids are preferred substrates. Possibly a synthetic activity of the lipase has to be assumed, resulting in an equilibrium of free and esterified derivatives. This effect was described by Partali (1992)..."(page 974, right column, to 975, left column).

One of ordinary skill in the art would conclude, from Breithaupt's statements, that conditions for enzymatic hydrolysis of fatty acid-esterified carotenoids from red pepper had been optimized.

In stark contrast to the prior art, enzymatic deesterification of fatty acid-esterified red pepper carotenoids according to the methods of the present invention surprisingly resulted in a preparation having a previously unattainable increased ratio of free to mono- and diester carotenoids. The enzymatic deesterification of fatty acid-esterified carotenoids of the present invention was compared to non-enzymatic, prior art methods (chemical saponification) based on the HPLC profiles of the extracted deesterification products. The results are presented in Examples 2-4 of the instant application. The novel improvements taught in the present invention include the addition of at least one of a cellulase, a protease, a pectinase, a suitable emulsifier, and/or addition of at least one metal ion (see Examples 2 and 3, page 49, line 17 to page 50, line 25) to the esterase reaction, followed by incubation of the source of fatty acid-esterified carotenoids for at least 2 hours, before extraction with ethyl acetate of the carotenoids in alkaline pH. As illustrated therein, it is abundantly clear that the novel and unique features characterizing the enzymatic deesterification of the present invention enable deesterification and recovery at least comparable (see Figure 4), and in some cases superior (see Figures 3, 5b and 5c) to that of the chemically saponified carotenoids (Figure 2). The HPLC profile presented in Figures 3, 5b and 5c demonstrate the capability of the method of the present invention to substantially deesterify all diester carotenoids, and nearly all mono-ester carotenoids, substantially eliminating fatty acid-esterified contamination of the free carotenoid more efficiently than chemical saponification. Such superior efficiency of deesterification of carotenoids has been previously unattainable, and is of significant advantage in preparing carotenoid pigments rich in free carotenoids having superior bioavailabilty and absorptive characteristics.

Further, the Examiner has stated that "employing recycled reagents is given no patentable weight". Applicant wishes to point out that, while reducing the present invention to practice, the use of recycled emulsifier in the extraction of the deesterified carotenoid-containing material was investigated, and found to result in surprisingly high rates of recovery. Further, it was surprisingly shown that matrix-bound, immobilized esterases can be recovered from the deesterification reaction mixture, and reused numerous times without significant loss of deesterification efficiency. Similarly, while reducing the present invention to practice, it was uncovered that the effluent liquid phase remaining after removal of immobilized esterase and solvent extraction of the carotenoid fraction following the enzymatic deesterification reaction of the present invention, contained considerable amounts of deoxycholate which, when dried, was reusable. As described in detail in the instant specification (see Example 6), the efficiency of deesterification of oleoresin carotenoids is not compromised by using recycled deoxycholate (see Figures 9a-9d), rather, an unexpected synergistic result was observed, actually increasing deesterification efficiency with repeated recycling of the deoxycholate (see, for example, Figures 9a-9d and 10).

The recycling of emulsifiers is neither trivial nor obvious, and required great effort to determine suitable conditions- the emulsifier could have remained in the carotenoid fraction after wash, and the carotenoids could have been removed in the washes. Following investigation of these parameters, optimal conditions for the lipase reaction and subsequent washes were determined.

As regarding the reuse of immobilized lipase, the recovery and reuse of recycled emulsifiers, such as deoxycholate, for deesterification of carotenoids is of great advantage not only for the improved simplicity and reduction of costs that it affords, but also for the greatly superior purity of effluent wastes from the deesterification process, which is of crucial environmental and ecological concern, especially where emulsifiers and detergents such as deoxycholate and Tween are involved.

Mild alkaline ethyl acetate extraction of deesterified carotenoids is taught in claims 120-122. The Examiner has rejected these claims, stating that the selection of a desired pH for extraction is obvious and lacking criticality, and within the range of one of ordinary skill of the art.

Applicant wishes to point out that while the standardization of a single parameter may be accomplished in routine, trial and error procedures, achieving the proper combination of conditions for efficient operation of a complex, multi-parametered process is not routine.

Applicant wishes to point out that, as was pointed out in the telephone interview of June 13, 2006, while reducing the present invention to practice, it was surprisingly uncovered that extraction of the de-esterified carotenoids in ethyl acetate under mild alkaline conditions results in superior, previously unattainable recovery of the de-esterified carotenoids. Ethyl acetate was chosen to allow greater efficiency of the extraction of the polar, deesterified carotenoids, and alkaline pH was chosen in order to titre the free fatty acids to a salt, for their efficient removal in the washes.

Figures 13a and 13b of the instant specification show the qualitative and quantitative advantage of ethyl acetate extraction. Whereas the HPLC chromatograms of the two enzymatically deesterified paprika oleoresin carotenoin preparations indicate an identical profile of highly deesterified carotenoids, the concentrations of carotenoids (162 mg carotenoids/1 g deesterified extract with ethyl acetate, compared with 12.5 mg carotenoids/g with hexane) is far greater with the mild alkaline ethyl acetate extraction. Figures 13c and 13d of the instant specification show the importance of mild alkalinization for efficient ethyl acetate extraction of enzymatically deesterified red pepper carotenoids. Whereas the HPLC chromatograms of the two enzymatically deesterified paprika oleoresin carotenoin preparations indicate an identical profile of highly deesterified carotenoids, the concentrations of carotenoids (210 mg carotenoids/1 g deesterified extract with mildl alkaline ethyl acetate extraction, compared with 74.9 mg carotenoids/g with ethyl acetate extraction without pH adjustment) is far greater with the mild alkaline ethyl acetate extraction.

In stark contrast, Breithaupt et al extract their carotenoid products with methanol/ethyl acetate/light petroleum (at a ratio of 1:1:1, pH 7.4), and fail to mention or imply mild alkaline ethyl acetate extraction of the carotenoids.

In order to illustrate the superiority of mild alkaline ethyl acetate extraction of esterified carotenoids, to that of the methods of Breithaupt, the instant inventors have undertaken a comparison of yield of total, and highly deesterified carotenoids from paprika oleoresin extracted by the mild alkaline extraction, compared to that of the

same material extracted by the methanol/ethyl acetate/light petroleum (1:1:1, pH 7.4) solvent of Breithaupt.

From the data brought in the accompanying Declaration, it is clear that the extraction of total carotenoids using alkaline ethyl acetate, as taught in the present invention, is at least three times as efficient as that of the prior art method of Breithaupt. Further, analysis of the extracted carotenoids revealed that the alkaline ethyl acetate extraction produces a carotenoid preparation more highly enriched in deesterified carotenoids.

Such differences are critical to the method of the invention. It will be appreciated that alkaline pH can reduce the number of washes by increasing saponification of the cleaved free fatty acids following enzyme treatment, facilitating their removal and reducing the number of wash steps, and volume of wash liquid required. The cost effectiveness of any process of fluid phase extraction of carotenoids is in part dependent on the volumes of wash water that are required, since the waste water often contains, inter alia, contaminating solvents. Thus, increased wash volumes lead to additional production costs, while reduced wash volumes reduce the burden of disposal of the contaminated wash water.

Thus, it is Applicant's strong opinion that referenced prior art does not anticipate the superior methods for enzymatic deesterification and extraction of fatty acid-esterified carotenoids of the present invention. However, according to the Examiner's recommendations made in the interview of June 13, 2006, in order to better define the present invention, and further distinguish it from the prior art, claim 95 has now been amended to include the limitations of original claims 102 and 120, now reciting:

> "A method of increasing a fraction of free carotenoids in a source of carotenoids in which at least some of the carotenoids are fatty acid esterified carotenoids, the method comprising the steps of
> (a) contacting the source of carotenoids with an effective amount of an esterase and a recycled emulsifier under conditions effective in deesterifying the fatty acid esterified carotenoids, wherein said conditions effective in deesterifying the fatty acid esterified carotenoids are characterized by addition of at least one additive selected from the group consisting of:
> a cellulose degrading enzyme;
> a protein degrading enzyme;
> a pectin degrading enzyme; and

at least one metal ion; and,

(b) extracting said source of at least partially deesterified carotenoids with ethyl acetate under alkaline conditions,

thereby increasing the fraction of free carotenoids in the source of carotenoids."

Thus, amended claim 95 now specifies the inclusion of additives selected from additional enzymes and a metal ion, and includes an additional step of alkaline ethyl acetate extraction, and as such is not and cannot be anticipated by the methods of Breihaupt.

35 U.S.C. § 103(a), Rejections

The Examiner has rejected claims 112-113 under 35 USC 103(a) as being unpatentable over the combination of Breithaupt (Z fur Naturforsch. 2000 55:971-75) in view of Kiy et al. (US Patent No. 6,350,890). The Examiner's rejections are respectfully traversed. Claims 102 and 120 have now been cancelled, rendering moot the Examiner's rejection thereof. Claims 95, 118, 121 and 122 has now been amended.

The Examiner has stated that Breihaupt fails to teach the use of immobilized lipase, and that Kiy et al. teach immobilized lipases for performing enzymatic reactions. The Examiner then concludes that using an immobilized lipase in the method of Breithaupt would have been obvious to one of ordinary skill in the art.

Regarding the methods of Breihaupt et al, the superiority of the claimed methods for enzymatic deesterification and extraction of fatty acid-esterified carotenoids of the present invention is described in detial hereinabove. Regarding Kiy et al., Applicant wishes to point out that Kiy et al. teaches methods of preparation and selective isolation of fatty esters and/or fatty acids from a biological source comprising reacting the biological source with an inert catalyst using continuous in-situ extraction, reaction chromatography, subjecting the inert catalyst to chromatography, and extracting the fatty esters or fatty acids from the desorbed and eluted reaction products. The inert catalyst taught by Kiy et al is solid inert aluminum oxide. Kiy et al does not teach the use of immobilized lipases for the isolation of fatty esters or fatty acids, rather, Kiy et al. teaches that the use of immobilized lipases for fat cleavage (cleavage of triglycerides), as has been taught in prior art, is undesirable as it has the disadvantage of low conversion rates with time (column 2). Thus, Kiy et

al teach away from the use of immobilized lipases for the extraction of fatty esters or fatty acids from a biological source.

Further, as recommended by the Examiner in the telephone interview of June 13, 2006, independent claim 95 has now been amended to include a step of alkaline ethyl acetate extraction of the deesterified carotenoids. Neither prior art documents teach alkaline ethyl acetate extraction.

Thus, it is Applicant's strong belief that one of ordinary skill in the art, in possession of both Breihaupt and Kiy, et al. would neither be motivated, nor able to employ immobilized lipases in the methods of Breihaupt to increase a fraction of free carotenoids in a source of esterified carotenoids as taught by the methods of now amended claim 95, and claims dependent therefrom.

The Examiner has rejected claims 102 and 114-119 under 35 USC 103(a) as being unpatentable over the combination of Breithaupt (Z fur Naturforsch. 2000 55:971-75) in view of Kanner et al. (in Fruchtsaft Union, 1984;18:219-25). The Examiner's rejections are respectfully traversed.

The Examiner states that Kanner et al teaches treating citrus peels with pectinases and cellulases, and extracting carotenoids with D-limonene. The Examiner then asserts that to employ one known extraction method such as taught by Kanner et al, and another known method such as taught by Breithaupt to produce free carotenoids would be obvious to one of ordinary skill in the art.

Regarding the methods of Breihaupt et al, the superiority of the claimed methods for enzymatic deesterification and extraction of fatty acid-esterified carotenoids of the present invention is discussed hereinabove. Regarding Kanner et al., Applicant wishes to point out that Kanner et al. fail to teach or mention the deesterification of carotenoids from citrus peel, or the extraction of deesterified carotenoids from a source and, as such, would not motivate one of ordinary skill in the art to produce free carotenoids by extracting at least partially deesterified carotenoids using treatment by pectinases and cellulases.

Further, independent claim 95 has now been amended to include a step of alkaline ethyl acetate extraction of the deesterified carotenoids. Neither prior art documents teach alkaline ethyl acetate extraction.

Thus, it is Applicant's strong belief that one of ordinary skill in the art, in possession of both Breihaupt and Kanner, et al. would neither be motivated, nor able

to employ pectinases and/or cellulases in the methods of Breihaupt to increase a fraction of free carotenoids in a source of esterified carotenoids as taught by the methods of now amended claim 95, and claims dependent therefrom.

35 U.S.C. § 112, 2nd paragraph Rejections

The Examiner has rejected claim 118 under 35 USC 112, 2nd paragraph as being indefinite for failing to point out and distinctly claim the subject matter of the invention. The Examiner's rejections are respectfully traversed. Claim 118 has now been amended.

Claim 118 now reads:

"118. The method of claim 1, wherein said protein degrading enzyme is ..."

Thus overcoming the Examiner's rejection thereof.

The Examiner has objected to the title under 35 USC 112 2nd paragraph as lacking sufficient description. The title of the invention has now been amended to read:

"Title: METHODS FOR EFFICIENT EXTRACTION OF CAROTENOIDS USING AN ESTERASE."

In view of the above amendments and remarks it is respectfully submitted that independent claim 95, and all claims directly or indirectly dependent therefrom are now in condition for allowance. Prompt Notice of Allowance is respectfully and earnestly solicited.

Respectfully submitted,

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Date: June 27, 2006.

Encl:
Petition for Extension (3 Months)
Declaration of Joseph Kanner
CV of Joseph Kanner